Spatially and genetically distinct African trypanosome virulence variants defined by host interferon-gamma response

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SPATIALLY AND GENETICALLY DISTINCT AFRICAN TRYPANOSOME VIRULENCE VARIANTS DEFINED BY HOST-INTERFERON-GAMMA RESPONSE

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Abstract

We describe two spatially distinct foci of human African trypansomiasis in Eastern Uganda. The Tororo and Soroti foci of *Trypansoma brucei rhodesiense* infection were genetically distinct as characterised by 6 microsatellite and 1 minisatellite polymorphic markers, and were characterised by differences in disease progression and host-immune response. In particular, infections with the Tororo genotype exhibited an increased frequency of progression to and severity of the meningoencephalitic stage and higher plasma IFN-γ concentration compared to those with the Soroti genotype. We propose that the magnitude of the systemic IFN-γ response determines the time at which infected individuals develop CNS infection, and this is consistent with the recently described role of IFN-γ in facilitating blood brain barrier transmigration of trypanosomes in experimental model infection. The identification of trypanosome isolates with differing disease progression phenotypes provides the first field-based genetic evidence for virulence variants in *T.b.rhodesiense*.

Keywords

African trypanosomiasis; interferon-gamma; virulence

Introduction

Human African trypanosomiasis (HAT), is caused by infection with the tsetse fly-transmitted hemoflagellates *Trypanosoma brucei rhodesiense* (in East and Southern Africa) and *T.b.gambiense* (in West and Central Africa). This disease is re-emergent with a current

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The authors have no commercial or other association which would pose a conflict of interest.

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estimate of over 300,000 cases and a further 60 million people at risk of infection [1]. The early (or haemolymphatic) stage of HAT commences 1-3 weeks after an infective fly bite with parasites proliferating within the blood and lymphatic system. Symptoms include general malaise, anemia, headache, pyrexia, weight loss and weakness. The late (meningoencephalitic) stage of infection coincides with the invasion of the CNS by parasites and is associated with psychiatric, motor, sensory and sleep disorders, eventually progressing to a final stage involving seizures, somnolence, coma, and death [2]. Typically *T.b.rhodesiense* infections are acute, while *T.b.gambiense* presents as a chronic disease [3]. However, within each sub-species there are differences in the rate of disease progression. In *T.b.rhodesiense* infection, large scale spatial differences in disease presentation have been described, where a “severe” and “mild” disease are associated with “northern” and “southern” disease foci respectively [4], and where the mild disease is associated with lower levels of systemic inflammatory response [5]. These differences in disease severity may result from variation in host-resistance, genetic variation in parasite virulence, socioeconomic and environmental factors, or a combination of all.

Progression to the late stage requires penetration of the blood-brain-barrier (BBB) by trypanosomes. Recent animal model studies have demonstrated that IFN-γ plays a critical role in this process, by modulating endothelial basement membrane laminin expression and lymphocyte transmigration [6]. Both clinical and experimental animal studies have observed systemically high levels of IFN-γ during trypanosome infection [7-9], and following trypanosome invasion of the CNS there is direct relationship between the severity of neuropathology and expression of IFN-γ in the brain [10].

In this paper we describe two spatially distinct HAT foci in Eastern Uganda, with genetically distinct parasites, allowing us to test the hypothesis that disease progression is determined by parasite genotype and host-IFN-γ response.

Materials and Methods

Study Sites and Subjects

Patients with HAT and non-infected control individuals presenting to local hospitals or identified during community surveillance were recruited in Eastern Uganda in 2002 and 2003. The Tororo, Iganga, Jinja and Busia Districts define a common ecotope for the transmission by *Glossina fuscipes fuscipes* of *T.b.rhodesiense* which will be referred to henceforth as the Tororo focus, while the Soroti District contains a separate *G.f.fuscipes* ecotope where HAT emerged as a new epidemic in 1998/9 [11]. Diagnosis was by microscopic detection of trypanosomes in wet blood films, giemsa stained thick blood films or in the buffy coat fraction after microhematocrit centrifugation [12]. Following admission, a detailed physical examination was performed and neurological involvement was assessed using the Glasgow Coma Score (GCS) [13]. The GCS gives a measure of the degree of impairment of consciousness, with a score of 15 as normal. The ranges 14-12, 11-8, < 8, indicate mild, moderate and severe impairment of consciousness respectively. A clinical history was taken either from the patient or the attendant relative, and patients were classified on the basis of language to either Bantu, Western- or Eastern-Nilotic ethnic groups [14]. Stage determination was by examination of cerebrospinal fluid (CSF) using the WHO criteria in which patients with trypanosomes in the CSF and/or a cell count >5cells/mm^3 were classified as late stage [15]. Early stage infection was treated with suramin and late stage infection with melarsoprol [9]. Subjects or their guardians signed consent forms after receiving standard information in their local language. Protocols were approved by the Grampian Research Ethics Committee (Aberdeen) and the Ministry of Health (Uganda). Malaria-parasitemic and microfilaremic individuals were excluded from the study.

Blood samples taken before treatment commenced were collected into EDTA-vacutainers (Greiner, Stroud, UK) and centrifuged for 10 minutes at 3000g. Platelet-depleted plasma was aliquoted and frozen immediately in liquid nitrogen. CSF samples taken as part of normal stage diagnosis were also frozen and stored in liquid nitrogen. Trypanosome DNA from a subset of cases was sampled by applying a 200μl suspension taken from the buffy coat layer to FTA cards (Whatman Bioscience, Maidstone, UK), which were dried and stored at room temperature. Cases were selected to include representatives from each village and no more than one case per compound/family.

Cytokine Assays

IFN-γ, IL-1β, IL-6, TGF-β and IL-10 concentrations were measured using a solid phase sandwich ELISA (OptiEIA, BD Pharmingen, Cowley, UK) as described previously [16]. Biological limits of detection for these assays were 1.8, 10.2, 8.3, 19.2 and 1.6 pg/ml respectively.

FTA filter preparations and whole genome amplification

Discs of 1.2 mm diameter were cut out of each blood spot using a Harris Micro-punch™ (Whatman). The discs were washed three times with 200 μl FTA purification reagent (Whatman), and twice with 200 μl 1 mM TE buffer pH 8.0, with incubation for 5 minutes at each wash. The washed discs were air-dried for an hour, and then used as substrate for whole genome amplification reactions.

Whole genome amplification was carried out using Multiple Displacement Amplification (MDA) technology directly on washed FTA punched discs [17]. Three independent reactions were carried out for each sample and products were stored at −20°C.

PCR-based genotyping

One μl of each MDA product was used as template for PCR, in a volume of 20 μl. The microsatellite loci, ch1/18, ch2/5, ch2/PLC, ch3/5L5, ch4/M12C12, ch5/JS2 and minisatellite locus, ch3/292, used in this study have been described previously [18, 19]. Both outside and nested PCR primers for each marker are detailed in Table 1.

PCR conditions were: PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 μM EDTA, 113 $\mu g.ml^{-1}$ BSA, 1 mM of each four deoxyribonucleotide triphosphates), 1 μM of each oligonucleotide primer, and 1 unit of Taq polymerase [Abgene] per 20 μl reaction, 2.5 U per 50 μl reaction. For the nested reactions, 1 μl of a 1/100 dilution of first round product was used as template in the second round PCR. PCR products were resolved by electrophoresis on a 3% Nusieve GTG® agarose gel (Cambrex), stained with ethidium bromide and visualised under UV light.

Allele Size Determination

The number and size of alleles amplified in each sample was determined by using one fluorescently labelled primer for each locus (FAM) followed by separation of products using a capillary-based sequencer (ABI 3100; Applied Biosystems). DNA fragment size was determined relative to a set of ROX-labelled size standards (GS500 markers; Applied Biosystems) using Genescan© software, which allowed resolution to the level of 1 bp.

For the minisatellite locus, Ch3/292, allele band sizes were determined on the basis of mobilities relative to a reference standard lane (restriction fragments of λ *Hin*dIII and φ×174 *Hae*III) as described in [20].

Genetic analysis

The programs clustering calculator [21] and Treeview [22] were used to generate a dendrogram in which multilocus genotypes were compared using unweighted arithmetic average as the clustering method and a pairwise distance matrix of the multilocus genotypes (Jaccard's similarity coefficient) for the input data. This allowed the analysis of the similarity between each multilocus genotype to be determined. The genetic data analysis (GDA) program [23] was used to determine Nei's genetic distance (D) [24] and Wright's fixation index (Fst) [25] between each group of isolates from different foci.

Statistical Analyses

The statistical package JMP5 (SAS Institute, Cary, North Carolina) was used. The distributions of cytokine concentrations were all right skewed. After log transformation all approached normality. Log transformed cytokine values were compared using appropriate parametric tests as indicated in the results using the linear modelling platform in JMP. Distributional assumptions were tested using Shapiro-Wilks test and Levene's test for homogeneity of variance. Age was stratified 0-5; 6-14; 15-29;and 30+ years. Categorical variables were compared using Pearson's χ^2 or Fisher's Exact test.

Results

Distinct genetic variants cause disease in Tororo and Soroti Districts

One hundred and forty four patients with HAT were recruited during the period January 2002 to August 2003, of whom 115 came from the Soroti focus and 29 from the Tororo focus. Parasite DNA, from a subset of 22 subjects in the Tororo focus and 27 subjects in the Soroti focus, was subject to whole genome amplification followed by genotyping with 6 microsatellites and 1 minisatellite marker, using nested PCR. The multilocus genotype of each sample was determined based on the combination of alleles at the 7 loci, revealing 30 distinct multilocus genotypes. To determine the relationship between the isolates, a dendrogram based on Jaccard's similarity index was constructed (Fig. 1). A *T.b.rhodesiense* sample, isolated from a sleeping sickness patient in Malawi in 2002 (NKK/T/02-02, [5]) was also included in this analysis for comparison. Two distinct clusters of genotypes (groups 1 and 2, Fig.1) were identified using this approach, and the bootstrap value for the node that separates the two groups is high (97 of 100 replications). Group 1 genotypes contains only isolates from Soroti whereas group 2 contain only isolates from Tororo, clearly demonstrating that there are distinct parasite genotypes which are responsible for sleeping sickness in the two individual foci. This was confirmed by measurement of Nei's genetic distance between the groups (0.132).

Disease characteristics of Tororo and Soroti HAT subjects

The disease characteristics at each focus are summarised in Table 2. While there were no significant differences in parasitemia and anemia between the two foci, a significantly higher proportion of patients had progressed to the late stage in the Tororo focus at the time of diagnosis. There was a higher frequency of hepatomegaly and splenomegaly and also a small but significant increase in body temperature observed in patients from the Tororo who also exhibited a significantly higher frequency of moderate (GCS 9-11) or severe impairment (GCS 8 or less) of consciousness. As this result could be influenced by the higher proportion of late stage patients in Tororo, the frequency of moderate or severe impairment of consciousness was also compared for late stage cases only. Again, a significantly higher frequency of patients with GCS less than 12 was found in Tororo as compared to Soroti. Despite the apparently more advanced disease presentation in the Tororo focus, there was no significant difference in the duration of illness as reported by

patients. There were differences in age and gender composition in Tororo and Soroti, however these factors were not significantly (P>0.3) associated with infection stage.

Tororo and Soroti foci are characterised by differences in plasma IFN-γ and IL-6 concentration

The plasma concentrations of a panel of cytokines involved in the inflammatory response and its regulation were then measured (Figure 2). The concentrations of IFN-γ in Tororo, and IL-6, IL-10, IL1-β and TGF-β in both Soroti and Tororo were elevated over control levels in HAT patients, and furthermore there were significant differences in the concentrations of IFN-γ (P<0.0001, unpaired t-test), IL-6 (P<0.05) between the two foci. TNF-α concentration was not significantly increased in either patient group. As blood samples from a subset of Tororo and Soroti patients had been used for parasite-genotyping (Fig. 1), a further analysis was carried out in which the plasma cytokine levels of genotyped-infections only was measured. The results from this analysis were entirely consistent with those from the full Tororo and Soroti study cohorts (Table 3). For comparison the relevant plasma cytokine data for Malawi patients published in [5] is also presented in Table 3.

As the Tororo and Soroti groups differed in age and gender structure (Table 2), these factors were tested for effects on IFN-γ and IL-6 concentration. While age had no significance as an explanatory variable, female patients from Soroti showed a significant increase in plasma IFN-γ concentration (Female HAT patients: geometric mean (95% CI) 78.2 (60.9-102.5) pg/ml; Male patients 41.7 (30.8-56.2) pg/ml; P<0.05 unpaired t-test). No similar gender difference was evident in the Tororo patients. Because of the apparent gender effect on IFN-γ levels in Soroti, cytokine data were reanalysed using age and gender matched pairs of cases. Each of the 29 Tororo patients was paired with a Soroti patient of the same gender and within 5 years of the same age. Where more than one patient met these criteria, the pair with the closest diagnosis date was selected. In this subset of matched cases, IFN-γ and IL-6 concentrations continued to differ between the groups (Geometric mean (95% CI); IFN-γ Tororo 262.4 (179.5-383.7) pg/ml; Soroti 68.0 (48.9-94.6); P<0.001, paired t-test; IL-6 Tororo 76.7 (47.5-123.9) pg/ml; Soroti 27.9 (16.3-44.7) pg/ml P<0.01, paired t-test).

IFN-γ response is independent of subject ethnicity

Analysis of plasma IFN-γ levels in the aggregated Tororo and Soroti patient groups suggested that ethnolinguistic group was a significant determinant of response ($F_{2,137}$=12.6 P<0.0001), with the lowest concentrations in Eastern Nilo-Saharan patients (Table 4). However 99% of the Soroti subjects (Table 2) were of Eastern Nilotic background, and thus the apparent influence of ethnicity on IFN-γ could be confounded by the predominance of this one ethnic group. This interpretation was supported by the observation that, when only patients from the Tororo focus (n=29) were analysed, ethnicity had no effect on plasma IFN-γ concentrations (Table 4).

As a further test for the effects of ethnicity on IFN-γ concentration, the 6 Eastern Nilotic subjects from the Tororo focus were age and sex matched with 6 Eastern Nilotic subjects from Soroti. In this subset of cases, there continued to be a significant difference in plasma IFN-γ concentration between the groups (Soroti geometric mean (95% CI) 60.3 (27.1-134.2) pg/ml; Tororo 365.0 (221.4-544.6) pg/ml; P<0.05 paired t-test). Plasma IL-6 did not vary according to subject ethnicity.

CNS IFN-γ is associated with moderate and severe coma

Trypanosomiasis patients from Tororo and Soroti did not exhibit any significant difference in CSF IFN-γ concentration (Table 5). As there was an increased frequency of patients with moderate (GCS<12) and severe impairment of consciousness (GCS<8) in Tororo, the

relationship of CSF IFN-γ concentrations to GCS was investigated. While there was no significant effect of GCS on CSF IFN-γ in Soroti, in Tororo patients with a moderate (n=5) and severe (n=3) impairment of consciousness exhibited a significantly higher concentration of CSF IFN-γ (Table 5) compared to patients with GCS scores of 12-14 (mild impairment of consciousness, n= 6) and 15 (normal, n=15).

Discussion

HAT caused by *T.b.rhodesiense* presents a spectrum of clinical profiles, from acute infection rapidly progressing to the lethal meningoencephalitic stage to more chronic infections which, similar to *T.b.gambiense*, take several months or more to progress to CNS infection [4]. It has been proposed that such differences in disease tempo may be controlled by either parasite genotype or host genotype or a combination of both [5]. Spatial genetic variation of parasite populations certainly does occur [26] but its relationship to pathology has hitherto been unclear.

In 1998, the first cases of a new epidemic of sleeping sickness emerged in Soroti District [11], some 150km North of the historic Tororo focus and between 2002 and 2003 we carried out a cross-sectional survey of cases in both foci. Analysis of clinical histories indicated a difference in disease virulence between the two foci, with Tororo subjects showing increased progression to the CNS infection stage. While this could have been the result of a longer period of illness prior to admission, patient interview data indicate no significant difference in duration of disease between Tororo and Soroti. This suggests that the more advanced progression in Tororo is not simply the result of a longer duration of disease. The difference in clinical profile between these spatially distinct disease foci allowed us to test the hypothesis that disease progression and thus virulence in sleeping sickness was determined by parasite genotype. On the basis of the difference in allele type and multilocus genotype, we have shown that the geographically separated *T. brucei* populations of Soroti and Tororo, isolated at the same time and from the same host type, are genetically distinct, providing evidence for a lack of gene flow between these populations. This conclusion was supported by a moderate genetic distance between the populations. Thus we demonstrate for the first time that distinct parasite genotypes circulate in spatially separated foci and are associated with differences in virulence as measured by progression to and severity of the CNS infection stage.

It has previously been demonstrated in experimental HAT models that pathology is associated with the systemic [27] and CNS production [10] of inflammatory cytokines. We therefore investigated the relationship between systemic cytokine response and difference in clinical presentation between patients in Tororo and Soroti. There was a generalised upregulation of plasma IL1β, TGF-β and IL-10 in all patients, consistent with previous studies in *T.b.rhodesiense* [16] and *T.b.gambiense* [28, 29]. However, IFN-γ, and IL-6 were detected at consistently different concentrations in the plasma of Tororo subjects compared with Soroti subjects, after controlling for differences in age, gender and ethnicity between the two foci. IFN-γ concentration was higher in Tororo patients, while IL-6 concentration was higher in Soroti patients. Studies in mouse model infections have demonstrated that IFN-γ plays a pivotal role in the host response to trypanosomiasis. It is detected in splenic T-cells within 24h of infection [30], reaches higher plasma concentrations with laboratory trypanosome strains of increasing virulence [7], and appears to be derived from both the innate cellular immune system [31] and adaptive responses by T-cells to variant surface glycoprotein epitopes [32], trypanin [33] and other undefined parasite-antigens. IFN-γ contributes to pathogenesis through macrophage activation and resulting nitric oxide and TNF-α production [27, 34, 35]. However, IFN-γ appears to be essential for the control of parasitemia in mouse models [36-38]. For the present study, of particular relevance is the

recent finding in the mouse model that IFN-γ is a regulator of the blood-brain barrier, which trypanosomes must cross in order to initiate the CNS infection [6]. Mice genetically manipulated to be deficient for IFN-γ and the IFN-γ receptor all showed impaired and delayed invasion of the brain parenchyma with an accumulation of parasites between the endothelial and parenchymal basement membranes. If these findings also apply to human disease, individuals responding with high levels of IFN-γ would be expected to show increased progression to CNS infection, and this is the case in the Tororo focus in this study. HAT cases in Tororo also showed a higher frequency of moderate and severe impairment of consciousness as measured by GCS compared to Soroti. In mouse models, upregulation of IFN-γ production in the brain parenchyma is associated with the onset and increasing severity of neuroinflammation [10, 39], leading to the neurological sequelae of sleeping sickness. When the level of IFN-γ in the CSF of patients was investigated, and it was found that in Tororo sleeping sickness cases patients with GCS less than 12 exhibited significantly higher levels of CSF IFN-γ. Thus we provide the first clinical evidence of an involvement of IFN-γ in the initiation and severity of meningoencephalitic sleeping sickness.

The significance of the higher concentration of plasma IL-6 in Soroti patients is not clear. IL-6 is a multifunctional cytokine with pro-inflammatory characteristics, but also acts as a neuroprotective factor [40]. While IL-6 has previously been shown to be expressed at high levels both in the peripheral circulation and in the CNS during experimental mouse [10, 41] and human trypanosomiasis [16], the functional relationship between IL-6 and pathogenesis in HAT requires further investigation.

Plasma IL-10, IL-1β and TGF-β were elevated at similar levels in both Tororo and Soroti. In a previous study contrasting mild and severe HAT in Malawi and Uganda respectively, it was proposed that higher levels of plasma TGF-β observed in mild cases may point to a role of this cytokine in regulating inflammatory pathology [5]. While in the present study, TGF-β was not related to disease severity, when compared to the cytokine responses reported in [5], both Tororo and Soroti patient plasma TGF-β levels were lower than in Malawi HAT patients, while at the same time Tororo patient plasma IFN-γ levels were significantly higher than those of Malawi patients, which were not significantly different to the Soroti patient plasma IFN-γ concentration. Thus, while the variations in disease severity we describe within Uganda appear to be related to differences in the IFN-γ response to infection, at larger spatial scales, such as those encompassing the mild and severe HAT symptoms of Southern and Northern areas of East Africa respectively, additional involvement of inflammatory pathology and its regulation by TGF-β may be involved. Field clinical studies of HAT are limited to cross-sectional design by ethical and logistical constraints, and therefore it is not possible to confirm causality or mechanism in the relationship between plasma and CSF cytokines and disease progression. The detailed dissection of the host-response to the Tororo and Soroti parasite genotypes and mechanistic role of IFN-γ in promoting CNS invasion and neuropathology now requires study in refined experimental mouse models [10] in which the invasion of the CNS and progression of CNS pathology may be measured.

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References

1. WHO. Control and Surveillance of African Trypanosomiasis. 1998; 881:1–113. [PubMed: 10070249] WHO Tech. Rep. Ser.
2. Kennedy PGE. Human African Trypanosomiasis of the CNS: current issues and challenges. J.Clin.Invest. 2004; 113:496–504. [PubMed: 14966556]
3. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S. The trypanosomiases. Lancet. 2003; 362:1469–80. [PubMed: 14602444]
4. Ormerod WE. Taxonomy of the Sleeping Sickness Trypanosomes. J.Parasitol. 1967; 53:824–830. [PubMed: 6035724]
5. MacLean L, Chisi JE, Odiit M, Gibson WC, Ferris V, Picozzi K, Sternberg JM. Severity of Human African Trypanosomiasis in East Africa is associated with geographic location, parasite genotype and host-inflammatory cytokine response profile. Infect Immun. 2004; 72:7040–7044. [PubMed: 15557627]
6. Masocha W, Robertson B, Rottenberg ME, Mhlanga J, Sorokin L, Kristensson K. Cerebral vessel laminins and IFN-gamma define *Trypanosoma brucei brucei* penetration of the blood-brain barrier. J Clin Invest. 2004; 114:689–94. [PubMed: 15343387]
7. Bancroft GJ, Sutton CJ, Morris AG, Askonas BA. Production of interferons during experimental African trypanosomiasis. Clin Exp Immunol. 1983; 52:135–43. [PubMed: 6190591]
8. Mabbott NA, Coulson PS, Smythies LE, Wilson RA, Sternberg JM. African trypanosome infections in mice that lack the interferon-gamma receptor gene: nitric oxide-dependent and - independent suppression of T-cell proliferative responses and the development of anaemia. Immunology. 1998; 94:476–80. [PubMed: 9767434]
9. MacLean L, Odiit M, Okitoi D, Sternberg JM. Plasma nitrate and interferon-gamma in *Trypanosoma brucei rhodesiense* infections: evidence that nitric oxide production is induced during both early blood-stage and late meningoencephalitic-stage infections. Trans R Soc Trop Med Hyg. 1999; 93:169–70. [PubMed: 10450441]
10. Sternberg JM, Rodgers J, Bradley B, Maclean L, Murray M, Kennedy PG. Meningoencephalitic African trypanosomiasis: Brain IL-10 and IL-6 are associated with protection from neuro-inflammatory pathology. J Neuroimmunol. 2005; 167:81–9. [PubMed: 16054238]
11. Fevre EM, Coleman PG, Odiit M, Magona JW, Welburn SC, Woolhouse ME. The origins of a new *Trypanosoma brucei rhodesiense* sleeping sickness outbreak in eastern Uganda. Lancet. 2001; 358:625–8. [PubMed: 11530149]
12. Woo PT. Evaluation of the haematocrit centrifuge and other techniques for the field diagnosis of human trypanosomiasis and filariasis. Acta Trop. 1971; 28:298–303. [PubMed: 4400769]
13. Teasdale G, Jennett B. Assessment of coma and impaired consciousness. A practical scale. Lancet. 1974; 2:81–4. [PubMed: 4136544]
14. Gordon, RGGjun. Ethnologue: Languages of the world. 15th ed.. Dallas: SIL International; 2005.
15. Cattand P, Miezan BT, de Raadt P. Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes. Bull World Health Organ. 1988; 66:83–6. [PubMed: 3260146]
16. MacLean L, Odiit M, Sternberg JM. Nitric oxide and cytokine synthesis in human African trypanosomiasis. J Infect Dis. 2001; 184:1086–90. [PubMed: 11574928]
17. Morrison LJ, McCormick G, Sweeney L, Likuefack ACL, Truc PC, Turner CM, Tait A, MacLeod A. The use of multiple displacement amplification to increase the detection and genotyping of *Trypanosoma* species samples immobilised on FTA filters. Am.J.Trop.Med.Hyg. 2007 in press. [PubMed: 17556624]
18. MacLeod A, Tweedie A, McLellan S, Taylor S, Hall N, Berriman M, El-Sayed NM, Hope M, Turner CM, Tait A. The genetic map and comparative analysis with the physical map of *Trypanosoma brucei*. Nucleic Acids Res. 2005; 33:6688–93. [PubMed: 16314301]
19. MacLeod A, Turner CM, Tait A. A high level of mixed *Trypanosoma brucei* infections in tsetse flies detected by three hypervariable minisatellites. Mol Biochem Parasitol. 1999; 102:237–48. [PubMed: 10498180]

20. MacLeod A, Tweedie A, Welburn SC, Maudlin I, Turner CM, Tait A. Minisatellite marker analysis of *Trypanosoma brucei*: reconciliation of clonal, panmictic, and epidemic population genetic structures. Proc Natl Acad Sci U S A. 2000; 97:13442–7. [PubMed: 11078512]
21. Brzustowski J. Clustering Calculator. 2002available at http://www2.biology.ualberta.ca/jbrzusto/cluster.php, accessed 1st February 2007
22. Page RDM. Treeview: An application to display phylogenetic trees on personal computers. Comp. Apps Biosciences. 1996; 12:357–358. [PubMed: 8902363]
23. Lewis PO, Zaykin D. GDA (Genetic Data Analysis): Computer Program for the analysis of allelic data. 200, available at http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php, accessed 1st February 2007
24. Nei M. Genetic distance between populations. American Naturalist. 1972; 106:283–292.
25. Wright, S. Evolution and Genetics of Populations. Vol. 2. Chicago: University of Chicago Press; 1984. p. 295
26. MacLeod A, Turner CM, Tait A. The detection of geographical substructuring of *Trypanosoma brucei* populations by the analysis of minisatellite polymorphisms. Parasitology. 2001; 123:475–82. [PubMed: 11719958]
27. Magez S, Truyens C, Merimi M, Radwanska M, Stijlemans B, Brouckaert P, Brombacher F, Pays E, De Baetselier P. P75 tumor necrosis factor-receptor shedding occurs as a protective host response during African trypanosomiasis. J Infect Dis. 2004; 189:527–39. [PubMed: 14745712]
28. Lejon V, Lardon J, Kenis G, Pinoges L, Legros D, Bisser S, N'Siesi X, Bosmans E, Buscher P. Interleukin (IL)-6, IL-8 and IL-10 in serum and CSF of *Trypanosoma brucei gambiense* sleeping sickness patients before and after treatment. Trans R Soc Trop Med Hyg. 2002; 96:329–33. [PubMed: 12174791]
29. Courtin D, Jamonneau V, Mathieu JF, Koffi M, Milet J, Yeminanga CS, Kumeso VK, Cuny G, Bilengue CM, Garcia A. Comparison of cytokine plasma levels in human African trypanosomiasis. Trop Med Int Health. 2006; 11:647–53. [PubMed: 16640617]
30. Bakhiet M, Olsson T, van der Meide P, Kristensson K. Depletion of CD8+ T cells suppresses growth of *Trypanosoma brucei brucei* and interferon-gamma) production in infected rats. Clin Exp Immunol. 1990; 81:195–9. [PubMed: 2143706]
31. Sternberg JM. Immunobiology of African trypanosomiasis. Chem Immunol. 1998; 70:186–99. [PubMed: 9509676]
32. Schleifer KW, Filutowicz H, Schopf LR, Mansfield JM. Characterization of T helper cell responses to the trypanosome variant surface glycoprotein. J Immunol. 1993; 150:2910–9. [PubMed: 8454863]
33. Hill KL, Hutchings NR, Grandgenett PM, Donelson JE. T lymphocyte-triggering factor of african trypanosomes is associated with the flagellar fraction of the cytoskeleton and represents a new family of proteins that are present in several divergent eukaryotes. J Biol Chem. 2000; 275:39369–78. [PubMed: 10969087]
34. Sternberg J, McGuigan F. Nitric oxide mediates suppression of T cell responses in murine *Trypanosoma brucei* infection. Eur J Immunol. 1992; 22:2741–4?. [PubMed: 1396977]
35. Schleifer KW, Mansfield JM. Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. J Immunol. 1993; 151:5492–503. [PubMed: 8228241]
36. Hertz CJ. Filutowicz H and Mansfield JM. Resistance to the African trypanosomes is IFN-gamma dependent. J Immunol. 1998; 161:6775–83. [PubMed: 9862708]
37. Namangala B, Noel W, De Baetselier P, Brys L, Beschin A. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. J Infect Dis. 2001; 183:1794–800. [PubMed: 11372033]
38. Mansfield JM, Paulnock DM. Regulation of innate and acquired immunity in African trypanosomiasis. Parasite Immunol. 2005; 27:361–71. [PubMed: 16179030]
39. Hunter CA, Gow JW, Kennedy PG, Jennings FW, Murray M. Immunopathology of experimental African sleeping sickness: detection of cytokine mRNA in the brains of *Trypanosoma brucei brucei*-infected mice. Infect Immun. 1991; 59:4636–40. [PubMed: 1718878]

40. Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernandez J, Molinero A, Campbell IL, Hidalgo J. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp Neurol. 2003; 181:130–48. [PubMed: 12781987]

41. Eckersall PD, Gow JW, McComb C, Bradley B, Rodgers J, Murray M, Kennedy PG. Cytokines and the acute phase response in post-treatment reactive encephalopathy of *Trypanosoma brucei brucei* infected mice. Parasitol Int. 2001; 50:15–26. [PubMed: 11267928]

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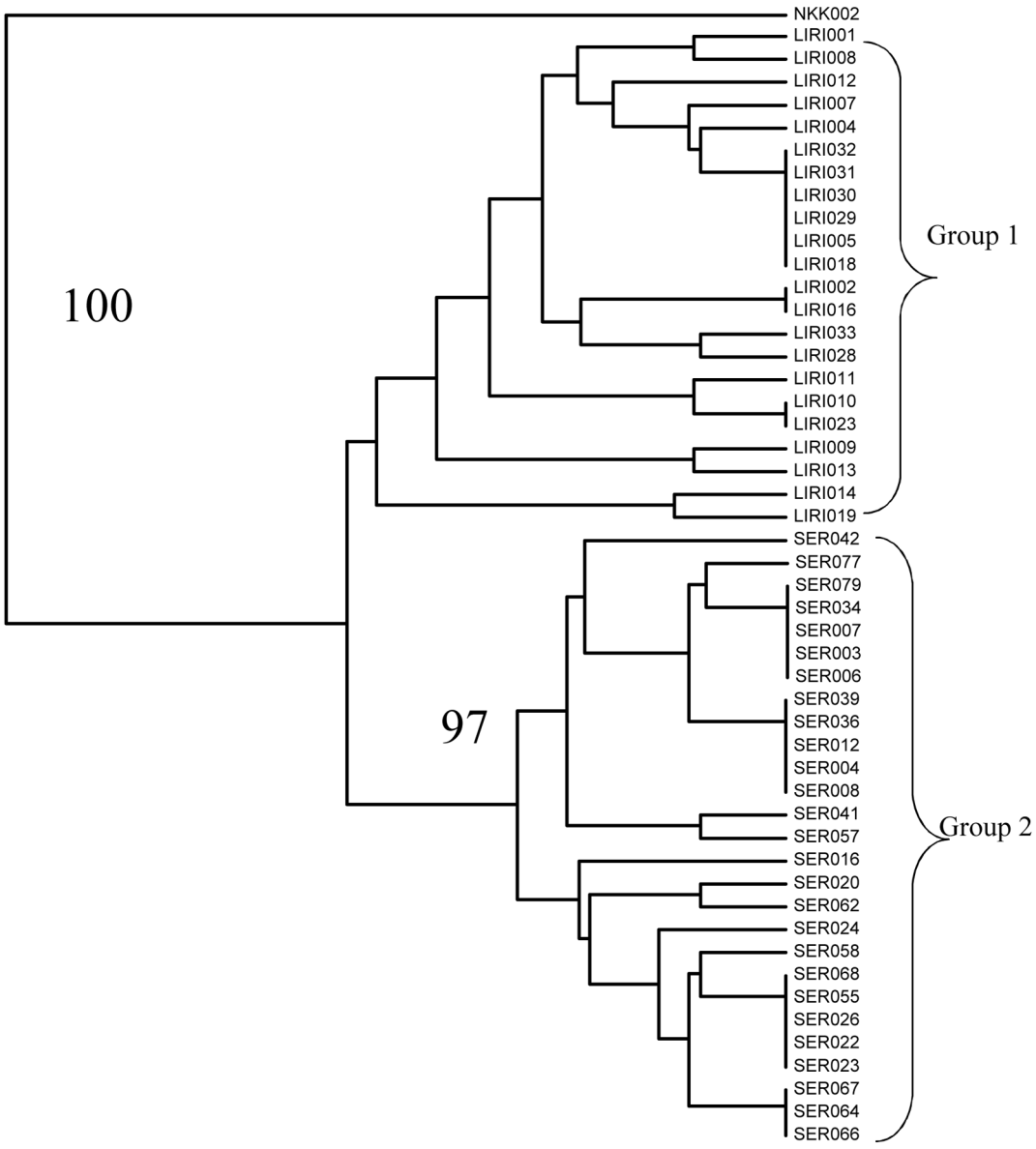

Figure 1 .

A dendrogram generated by an unweighted arithmetic average as the clustering method, showing the similarities between trypanosome multi-locus genotypes. Bootstrap values based on 100 replicates are shown for the main nodes. Groups 1 and 2 comprise the Tororo and Soroti genotypes respectively.

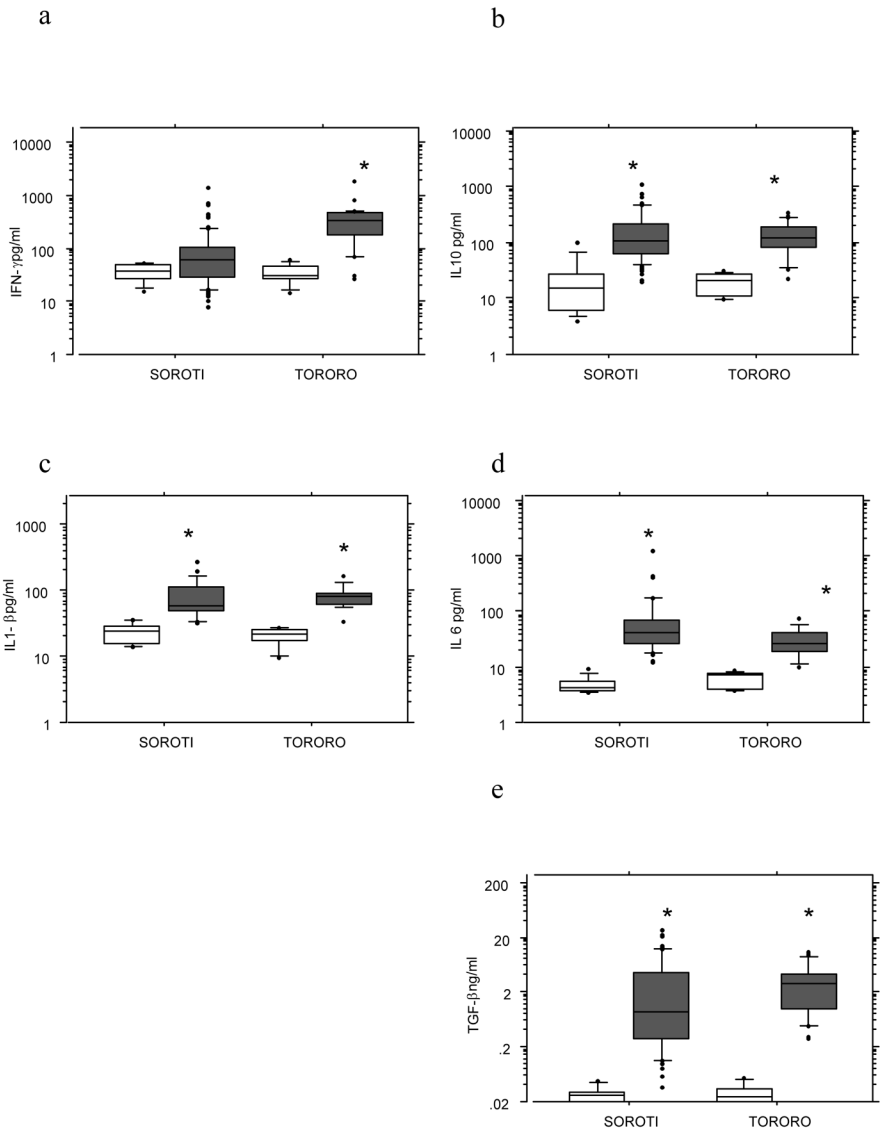

Figure 2.

Plasma concentrations of IFN-γ (a), IL-1β (b), IL-10 (c). IL-6 (d), and TGF-β (e) in patients (filled boxes) and control subjects (open boxes) in the Tororo and Soroti foci. Boxes show median and 25th and 75th percentiles. Whiskers show 10th and 90th percentiles. Dots represent outliers. *Significantly increased over control P<0.0001 Unpaired t-test on log transformed data.

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Table 1

Microsatellite outside and nested PCR primer sequences

Locus	Outside primers		Nested primers	
Ch1/18	1/18-C	tataatgcgtttgtgagaat	1/18-A-FAM	tgtgagaatggtactcacgcgctg
	1/18-D	gaagggagggaacagaagcaggg	1/18-B	caacgttagcacacaattcctgtg
Ch2/5	2/5-C	tatcgcggttatgtggatttgtgg	2/5-AFAM	atggcgtgtatcacattcgtgatg
	2/5-D	cacaacaaaactgccatgaggtac	2/5-B	ccgttggcattaggcacaagta
Ch2/PLC	PLC-G2	ttaagtggacgacgaaataacaaca	2/PLC-GFAM	caacgacgttggaagagtgtgaac
	PLC-H4	ttcaaacaccgtccccctcaataat	2/PLC-H3	ccactgacctttcatttgatcgctttc
Ch3/5L5/2	5L5/2-AA		3/5L5/2-AFAM	gtacgtggttaaccacaacctact
	gagcgtacattgcaggtagtgcgtagcg		2/5L5/2-B	ggaaactgcttaaacttgcgtgag
	5L5/2-C	acgaagaaacgaagcaaagaag		
Ch3/292	3/292-G1	cggaaaacgagggtgtgttacgcgac	3/292-G	gctgaacctgtgggcccctcaattg
	3/292-H	gctgaacctgtgggcccctcaattg	3/292-H2	acacccccctctccacttcagatac
Ch4/	M12C12-C	aaaacctcatccagtcgcactgg	M12C12-AFAM	tggacacacagaagcctaccg
M12C12	M12C12-B	taccctcatcaagtggtcg	M12C12-D	agtgtggtggtgcgtgcaaacttgg
Ch5/JS2	JS2-C	agtaatgggaatgagcgtcaccag	JS2-AFAM	gattggcgcaacaactttcacatacg
	JS2-D	gatcttcgcttacacaagcggtac	JS2-B	ctttcttccttggccattgttttactat

Table 2

Characteristics of sleeping sickness patients recruited in Tororo and Soroti Districts.

	All HAT cases	Soroti patients	Tororo patients
n	144	115	29
Age in years (median, (range))	22 (2-85)	20 (2-85)	25 (15-39)
Female %	56%	58.6%	44.8%
PCV (mean +/− SE)	29.7±0.5	29.5±0.6	30.7±0.9
Temperature (Median, IQR)	36.8 (36.4-37.2)	36.8 (36.4-37.0)	37.4 (36.8-38.0)[A]
% Late (meningoencephalitic) Stage	75.7	72.4	93.5[B]
%Glasgow Coma Score<12	13.3	6.9	37.9[C]
% Glasgow Coma Score <12 (meningoencephalitic cases only)	17.1	9.7	40.7[C]
Parasitemia[D] (Median, IQR)	0.5 (0-3)	0.4 (0-1)	1.5 (0-16.5)
Reported duration of illness (median days, IQR)	56 (22-86)	47 (21-67)	62 (30-102)
Lymphadenopathy %	41	43.1	33.3
Hepatomegaly %	4.1	1.0	14.8[B]
Splenomegaly %	15.2	10.4	31.0[B]
Ethnic composition	6.9;83.3;9.8	0.8,99.2,0	31;20.7;48.3
% Bantu; Eastern; Western Nilotic			

[A] Significantly increased over Soroti, $P<0.001$ unpaired t-test

[B] Significantly higher frequency in Tororo. $P<0.001$ χ^2 test

[C] Significantly higher frequency in Tororo. $P<0.05$ Fisher's exact test

[D] Parasites per 10 fields (at 400x) wet film

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Table 3

Plasma cytokine concentration (geometric mean (95%CI)) in HAT patients with genotyped parasite infections.

	Soroti (genotype 1) n=27	Tororo (genotype 2) n=22	Malawi HAT cases reported in ref [5]
IFNγ (pg/ml)[A]	74.4 (48.9-114.4)	254.6 (156.0-411.6)	56.8 (34.1-93.7)
IL-6 (pg/ml)[B]	79.8 (58.5-108.9)	51.9 (37.7-68.7)	121.5 (73.7-200.3)
IL-10 (pg/ml)[A]	115.6 (83.9-159.2)	130.3 (90.9-186.8)	134.3 (90.0-194.4)
IL-1β (pg/ml)[A]	76.7 (54.6-107.8)	79.8 (58.5-108.9)	82.2 (50.9-132.9)
TGF-β (ng/ml)[C]	1.58 (0.86-2.92)	2.51 (1.28-4.91)	6.0 (2.7-13.8)

[A] Tororo significantly increased over Soroti and previously reported Malawi {MacLean, 2004 #534} value $P<0.001$ (Unpaired t-Test)

[B] Soroti significantly increased over Tororo $P<0.05$ (Unpaired t-Test); Malawi significantly increased over Soroti and Tororo ($P<0.05$ Tukey HSD)

[C] Soroti and Tororo significantly lower than previously reported Malawi value ($P<0.001$)

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Table 4

Plasma interferon-γ and patient ethnicity.

	Interferon-γ pg/ml[A]	
Ethnicity	**All patients**	**Tororo patients only**
Bantu	183.0 (92.2-365.0), n=10	242 (121.5-483.2), n=9
Western Nilo Saharan	288.6 (148.4-555.6), n=14	290.0 (148.4-555.6), n=14
Eastern Nilo Saharan	64.1 (52.4-78.3), n=120[B]	270.4 (119.1-620.2), n=6

[A] Geometric mean (95% CI), n

[B] Significantly reduced compared to Western Nilo-Saharan and Bantu ($p<0.05$)

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Table 5

CSF Interferon-γ and Glasgow Coma Score in Tororo and Soroti patients

	IFN-γ (pg/ml) geometric mean (95% CI)	
	Tororo	**Soroti**
All patients	19.6 (13.3-29.1)	17.8 (14.7-21.5)
GCS12-15[A]	14.8 (9.0-24.5) n=21	17.4 (14.2-21.4) n=107
GCS<12[B]	32.0 (21.1-48.4) n=8[C]	22.6 (14.1-35.8) n=8

[A] GCS of 15 is normal. GCS of 12-14 is mild impairment of consciousness

[B] GCS of<12 is moderate impairment of consiousness, and GCS of 8 and below is coma.

[C] Significantly higher than in GCS 12-15 patients (P<0.01, unpaired t-test)